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BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF THE BROWNBANDED COCKROACH, *Supella longipalpa*, TO STEREOISOMERS OF ITS SEX PHEROMONE, SUPELLAPYRONE

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Abstract—Females of the brownbanded cockroach, *Supella longipalpa*, release a sex pheromone (supellapyrone) during a calling behavior and attract males from a distance. Supellapyrone has four possible configurations resulting from two asymmetric carbons at positions 2 and 4 (i.e., 2*R*,4*R*; 2*R*,4*S*; 2*S*,4*R*; and 2*S*,4*S*), but only the *RR* isomer is produced by females. Using pure synthetic stereoisomers in field tests, we showed that males are attracted to *RR* but also to high concentrations of the isomer *SR*. To study the activity of the stereoisomers in more detail we developed behavioral and electroantennogram (EAG) dose–response curves for each. Behaviorally, *RR* was the most active isomer with just 0.3 pg delivered on a filter paper being sufficient to elicit 50% male response in the olfactometer. Males were also attracted to *SR* and *SS* in the olfactometer, but at much higher dosages (100×) than the natural compound; *RS* did not elicit behavioral responses at any of the doses tested. In EAG assays, the antenna of male *S. longipalpa* showed high and similar sensitivity to *RR* and *SR*, but a much lower (10%) sensitivity to *SS* and practically no response to *RS*. The

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lack of agreement between behavioral and electrophysiological data suggested either that *RR* and *SR* stimulate different antennal sensory neuron types, or that some aspect of the interaction between the pheromone and the sensillum environment or the receptor neuron itself is different. To test the first hypothesis we examined the response of the antenna before and after adaptation with each of the four stereoisomers. Positive cross-adaptation between *RR* and *SR* suggests that these two compounds stimulate the same receptor cells. Therefore, the lack of agreement between behavioral and EAG dose–response curves could be explained by isomer-specific molecular interactions between the pheromone and the receptor neuron. Although *RR* and *SR* produced the same EAG amplitude, stimulation with *SR* resulted in a slower recovery rate (i.e., wider peaks) than stimulation with *RR*. To gain further understanding of the response specificity of the antennae to the different stereoisomers we compared EAG responses (amplitude and recovery time) in response to individual stereoisomers and binary mixtures of isomers. These tests showed additive responses of the EAG amplitude to mixtures of compounds, but nonadditive responses of EAG recovery time. Therefore, peak height and width are independent parameters of the EAG, probably representing different intrasensillar events, and likely resulting in the expression of different behavioral responses.

Key Words—*Supella longipalpa*, cockroach, Dictyoptera, sex pheromone, supellapyrone, chirality, electroantennogram, dose–response, EAG.

INTRODUCTION

Chemical communication with sex pheromones requires specificity of signals and receptors. Signal specificity is normally achieved by combinations of two or more pheromone components in specific blend ratios, whereas specificity of response normally requires the presence of several receptor neuron types, each responding to a different pheromone compound. A further refinement of the olfactory system consists of the ability to discriminate between absolute configurations of odor molecules. In communication systems that utilize stereoisomeric signal chemicals, the receptor system must also be stereochemically discriminating. Furthermore, in pheromone-mediated mate-finding in which reproductive isolation among species may be determined primarily by the chirality of a single pheromone component, it is not uncommon for responders to perceive antagonistic stereoisomers (Leal, 1998; Mori, 1998). Stereospecificity of odorants is probably more widespread in nature than earlier recognized, because only in recent years have new techniques routinely allowed resolution of trace amounts of enantiomers.

Females of the brownbanded cockroach, *Supella longipalpa* (F.) (Dictyoptera, Blattellidae), emit a volatile sex pheromone 5-(2′,4′-dimethylheptyl)-3-methyl-2*H*-pyran-2-one, or supellapyrone (Charlton et al., 1993), that attracts walking or flying males from a distance of several meters. This compound has four possible configurations at positions 2 and 4 of the alkyl side chain: (2*R*,4*R*), (2*R*,4*S*), (2*S*,4*R*), and (2*S*,4*S*). Electroantennographic detection coupled with chiral capillary gas chromatography (GC-EAD) has shown that the natural product is the

(2*R*,4*R*)-isomer (*RR*) and that male antennae also respond to the *SR*, but not to the *RS* or *SS* stereoisomers (Leal et al., 1995).

A mixture of supellapyrone stereoisomers effectively attracts *S. longipalpa* (Liang et al., 1998), but the attractant efficiency of the individual isomers has not been investigated. Synthesis of a mixture of stereoisomers is more economical than synthesis of the *RR* enantiomer, but whether the attractancy of *RR* in such a mixture is impaired by the presence of other stereoisomers is unknown. The four stereoisomers have been synthesized recently (Fujita and Mori, 2001), which prompted us to study the response of *S. longipalpa* males to each isomer and to their mixtures in the laboratory and in the field.

METHODS AND MATERIALS

Insects and Pheromones. The colony of *Supella longipalpa* was reared in plastic cages under a 12:12 (light:dark) photoregime at $27 \pm 1^{\circ}$C, and was provided with water and food (Purina Rat Chow # 5012, Purina Mills, St. Louis, MO) *ad libitum*. Newly-emerged adults were collected from the colony every 3–4 days, the sexes were placed in different containers under a reversed photoperiod, and males were used in tests ~30 days later. Stereoisomers of supellapyrone (*RR*, *SR*, *RS* and *SS* >99.9% chemically and diastoreomerically pure) were synthesized following the procedures of Fujita and Mori (2001). The enantiomeric excesses of *RR*, *SR*, *RS*, and *SS* were 97.6, 99.8, 98.4, and 95.6%, respectively, as determined on a Chiraldex GTA chiral capillary column (20-m × 0.25-mm × 0.12-μm; Chrompack, Raritan, NJ) operated at 142°C in a Hewlett-Packard 6890 gas chromatograph. The pheromone glands in tergites 4 and 5 (Schal et al., 1992) were dissected from 5 to 6-day-old females, extracted in CH_2Cl_2 for 24 hr, and the pooled extract was concentrated under N_2 to 1 and 0.1 female equivalents (FE) per 10 μl. All solutions were stored at -20°C in glass vials with Teflon-lined caps.

Field Test. Field trapping experiments were conducted in the Department of Genetics at North Carolina State University in December, 2001. Hexane-rinsed rubber septa (Thomas Scientific, Swedesboro, NJ) were loaded with 1 μg of *RR*, *SR*, *SS*, or *RS* in 50 μl CH_2Cl_2. Septa loaded with solvent only served as controls. In a second experiment, septa were loaded with 15 ng of *RR*, *SR*, or *SS*. Septa were placed individually in the bottom of 0.5-l glass jars, the inner wall of which was coated with petroleum jelly to prevent cockroaches from escaping. The jars were wrapped in a paper towel held in place by a rubber band to facilitate cockroach climbing. Placement of the traps in the room each day was based on a randomized complete block design so that each treatment was tested at least once in each trapping location during the test (experiment 1: $N = 5$; experiment 2: $N = 6$). Trapped cockroaches were counted and released in the room daily.

Olfactometer Tests. We used 16 olfactometers, each consisting of a clear Plexiglas tube (60 cm long × 3 cm ID). The olfactometers were connected symmetrically

to a vacuum pump, which was set to provide a wind velocity of 20 cm/s in each tube. The downwind end of the tube was covered with a metal screen (1-mmz mesh), and males were restrained 7 cm from the downwind end of the tube, which was covered with a metal screen (1-mm^2 mesh), by means of a metal screen gate (0.5-mm^2 mesh). Males were acclimated in the olfactometers 30–60 min before the stimulus was introduced. The pheromone (0.01–1000 pg) was applied to a 0.5-cm^2 filter paper disk ~30 min before the first individual was tested. Each disk was attached to a cork with an insect pin and kept inside a glass vial until used. New samples were prepared each day and used for a maximum of 6 hr. The stimulus was introduced through a 7-mm diameter orifice located at the top of, and 1.5-cm from the upwind end of the tube, and the filter paper was positioned 0.5 cm from the floor of the tube. Two fluorescent lights wrapped in red photographic filters were placed 50 cm above the olfactometers to facilitate observations, which took place during the second half of the scotophase, the period of maximum sexual activity in this species (Liang and Schal, 1990). A test sequence consisted of opening the gate, introducing the sample, and recording behavioral events with a microcomputer-based time–event recorder until the males left the cage or for up to 2 min if no response was observed. For each set of males placed in the olfactometers, one was tested with solvent, another with 0.1 FE, and the rest were tested with the four stereoisomers of supellapyrone. We tested the lowest doses first and gradually increased the doses throughout the day. The tubes were rinsed with tap water at the end of the day. Each naïve male was tested with a single treatment, then discarded. The percentage of males ($N = 29$–30) responding was analyzed using probit analysis and logistic regression of log-transformed data (SAS Institute, 2000).

Electrophysiology. Males were taken out of the rearing chamber during the scotophase shortly before being tested, anesthetized with a short pulse of CO_2 and one of their antennae was detached with fine forceps. The proximal end of the antenna was placed in the narrow end of a Pasteur pipette filled with cockroach saline BG-SSA (Kurtti and Brooks, 1976). Several terminal segments of the distal end of the antenna were excised, and this end was placed in a second capillary. Ag–AgCl wires, 0.5-mm diameter, connected the saline-filled capillaries to a Grass P-16 amplifier (Astro-Med, Inc., West Warwick, Rhode Island) with coaxial cables and BNC connectors. With this setup we experienced little environmental noise so no further shielding was necessary. The antenna was slightly curved between the electrodes, forming a horizontal arch, which was positioned within a 1-cm-diameter glass tube, and clean humidified air was passed continuously over the antenna at 1.5 l/min. The test samples were delivered through a perforated rubber septum that was fitted at the end of a lateral branch of the glass tube 8-cm upwind from the antenna. The signal was acquired through an A/D board installed in an HP5890 GC and recorded and analyzed with ChemStation software (Agilent Technologies, Palo Alto, CA).

Fresh samples were prepared daily. Each sample was loaded onto a folded rectangular filter paper (1.5 cm^2), which was placed into the wide section of a Pasteur pipette. Two militers of room air were taken into a calibrated glass syringe and delivered as a rapid puff to the antenna through the pipette containing the test compound. The calibration curve started with the solvent blank (CH_2Cl_2), followed by 1 FE and then the samples in ascending doses. Solvent and 1 FE were puffed every 2–3 test samples and at the end of the run. Each sample was puffed three times and the average constituted the experimental unit. A different syringe was used for each treatment (stereoisomer and dose) and the syringes, along with all glass components of the apparatus were rinsed daily with acetone. Each treatment (isomer $\times$ dose) was tested on six different antennae. To control for variation among antennae, the average amplitude of each set of three responses was divided by the average amplitude in response to the previous 1 FE sample. Comparison among treatments was performed on square-root-transformed data using a general linear model (SAS Institute, 2000).

Cross-Adaptation EAG. Adaptation of antennae using several stimuli was tested. After recording the response of an antenna to the test stimuli (four isomers, solvent, and the general odorant geraniol (four isomers, solvent, and the general food odorant geroniol [Aldrich, Milwaukee, WI]) a filter paper containing the adapting odor also was placed in the 1.5 l/min airstream flowing continuously over the antenna. The response of the antenna to puffs of test stimuli was then recorded in the presence of the adapting odor. We used concentrations of adapting odors (1 ng of *RR* and *SR*, and 10 ng of *SS* and *RS*) that caused some adaptation but did not impair antennal response, and concentrations of test stimuli (10 ng of *RR* and *SR*, and 100 ng of *SS* and *RS*) that produced discernible EAG responses under these conditions. Pipettes and syringes were reused for several days and were kept at -20°C between tests. The section of the pipette exposed to the adapting stimulus was rinsed with acetone after each set of three puffs. For delivery of the puffs in this and the following test, we used a straight 10-cm-long glass tube with a single orifice 3 mm in diameter and 6-cm upwind from the antenna preparation.

An adaptation index (AI) was calculated such that AI = postadaptation ratio/preadaptation ratio, where the postadaptation ratio = EAG amplitude of test stimulus/geraniol after introduction of the adapting stimulus, and the preadaptation ratio = EAG amplitude of test stimulus/geraniol before the introduction of the stimulus. The lower the AI the higher the effect of the adapting stimulus, with an AI = 1 meaning no adaptation. The data were square-root-transformed and analyzed with ANOVA followed by a planned means comparison test (Tukey's test) (N = 7–8).

Individual Compounds and Mixtures. To determine the effect of the interaction between *RR* and *SR* on the response of antennae (peak width at half height and peak amplitude) we performed EAGs using 10 μl CH_2Cl_2 (negative control), 1 FE (positive control), 5 ng of *RR*, 5 ng of *SR*, a mixture of 5 ng of *RR* plus 5 ng of *SR*, 10 ng of *RR*, and 10 ng of *SR*. Six independent sample loadings were tested

on 18 antennae ($N = 17$–18). Peak height (mV) and peak width at half height were measured and normalized by the response to 1 FE. The data were square-root-transformed and differences among treatments were analyzed with ANOVA followed by a planned means comparison test (Tukey's test).

RESULTS

Field Tests. In Experiment 1 (1 μg per septum), traps baited with *RR*, *SR*, *SS*, *RS*, and solvent control trapped 10, 11, 0, 1, and 1 males, respectively. One ootheca-carrying female was collected in a trap baited with *RS*. In Experiment 2 (15 ng per septum), traps baited with *RR*, *SR*, *SS*, and control trapped 8, 0, 0, and 0 males, respectively. Thus *RR* was most attractive and *SR* also attracted males at high doses, whereas *SS* and *RS* were no different from the solvent control.

Behavioral Dose–Response Studies. Insects were generally quiescent at the start of each behavioral assay, and males that were not quiescent were not tested. The percentage of males responding increased with the dose of the test stimulus. *RR* elicited the highest percentage of responses at all concentrations and reached a plateau at ~0.003 ng (Figure 1). Males were 100-fold less responsive to *SR* and *SS*, and did not respond to any amount of *RS* or to the solvent control. *RS* was, therefore, excluded from statistical analyses. The response curves for *RR* and *SS* were not different from each other ($\chi^2 = 0.84$, df $= 1$, $P = 0.36$), but differed from *SR* ($\chi^2 = 4.8$, df $= 1$, $P = 0.028$), indicating similarity in curve shape for *RR* and *SS*. The 95% fiducial limits for the 50% response level of *RR* (0.0002–0.0015 ng) did not overlap with the fiducial limits of *SR* (0.005–0.087 ng) or *SS* (0.032–0.088 ng), but there was overlap between these last two treatments (Figure 1).

EAG Dose–Response Curves. EAG responses increased with stimulus strength (Figure 2). *RR* elicited the highest EAG amplitudes, followed by *SR*. Unlike the behavioral results, EAG responses to *SR* and *SS* differed. *SR* elicited higher EAG amplitudes than *SS* throughout the range of doses tested. Overall, EAG amplitudes with *SS* and *RS* as a group were much lower than EAG amplitudes with *RR* and *SR* as a group ($F = 12.53$, df $= 1, 130$, $P < 0.001$), but *RR* and *SR* had similar peak amplitudes ($F = 1.51$, df $= 1, 66$, $P = 0.22$). Lack of significant interaction between concentration and isomer in this pair ($F = 0.07$, df $= 1, 66$, $P = 0.79$) indicated that the slopes of *RR* and *SR* were similar. The EAG amplitude of *RS* was indistinguishable from the solvent control at all doses except the highest.

Although the EAG responses to *RR* and *SR* had similar amplitudes at equivalent concentrations, the EAG deflection from *SR* had a much slower recovery time than the EAG response to *RR* (Figure 3, inset; $F = 121.43$, df $= 1, 60$, $P < 0.001$), and consequently had a significantly lower ratio of peak amplitude to peak width than *RR* (Figure 3; $F = 47.41$, df $= 1, 60$, $P < 0.001$). EAG amplitude increased

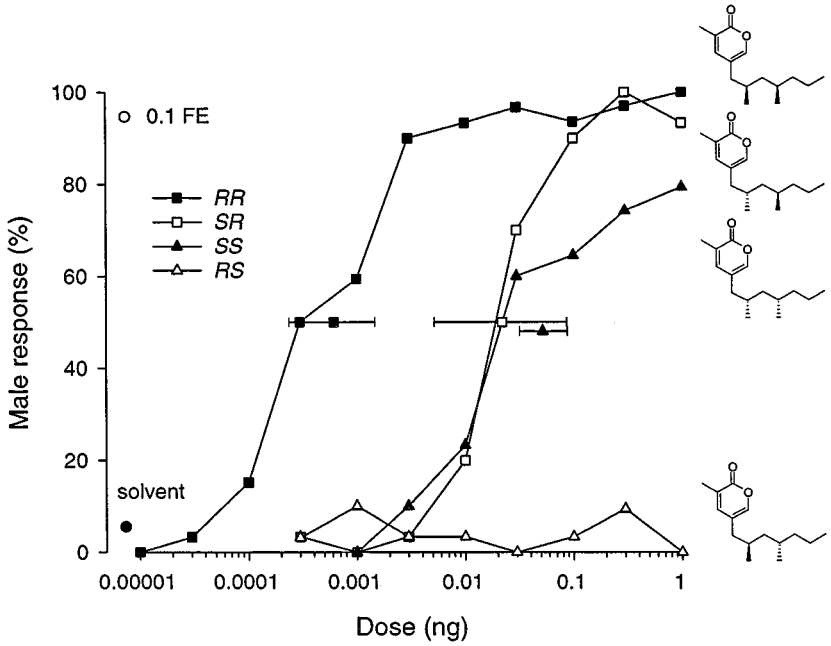

FIG. 1. Dose–behavioral response curves of *S. longipalpa* males to the four stereoisomers of supellapyrone. The percentage of males that started walking toward a source of supellapyrone, 0.1 female equivalents (FE), or a solvent control in an olfactometer was recorded ($N = 29$–30). Horizontal error bars indicate 95% fiducial limits for the 50% response levels to *RR*, *SR*, and *SS* determined from probit analysis (*SS* and *SR* fiducial limits have been separated for convenience). The symbol in the middle of the fiducial limits line marks the 50% response of the predicted response curves.

more with dose than did recovery time, and while this trend was steeper for *RR* than *SR* the slopes for these two isomers were marginally nonsignificant (no isomer $\times$ concentration interaction: $F = 2.24$, df $= 5, 60$, $P = 0.06$)

Cross-Adaptation EAG. The introduction of adapting odorants or the solvent control into the EAG air stream resulted in cross-adaptation among some of the compounds (Figure 4). Antennae exposed to a continuous stimulus of geraniol became less responsive to this compound but their responsiveness to the other compounds did not diminish (AI > 1, no difference among treatments; $F = 0.96$, df $= 5, 24$, $P = 0.46$). (*RS*)-supellapyrone and the solvent, on the other hand, did not cause any adaptation to any compound (AI ≈ 1, no difference among the treatments; $F = 1.22, \text{df} = 5, 37, P = 0.33$ and $F = 2.03, \text{df} = 5, 42, P = 0.094$, respectively). The *RR*, *SR*, and *SS* isomers adapted the antenna to themselves, to each other, and to 1 FE of natural supellapyrone (AI < 1), but not to solvent or

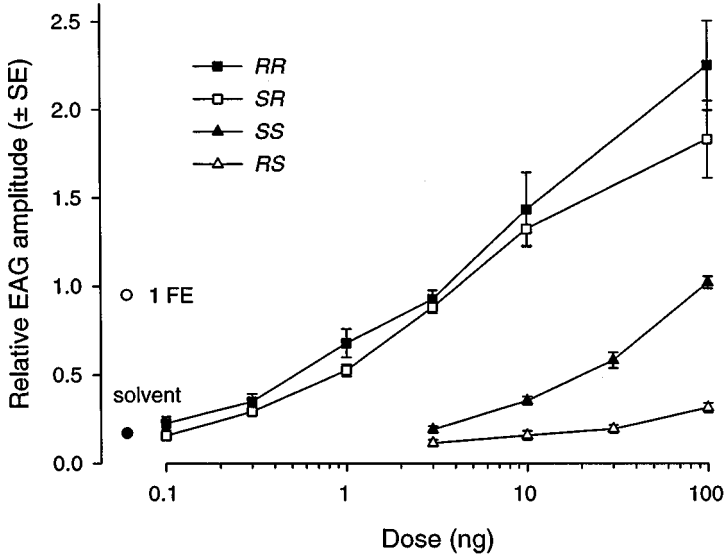

FIG. 2. Electroantennogram responses (mean ± SE) of *S. longipalpa* males to the four supellapyrone stereoisomers, 1 FE of extract, and a solvent control. To standardize the response of the antennae the amplitude generated by each test sample was divided by the amplitude generated by an extract of tergites 4 and 5 of a virgin female (1 FE).

to the *RS* isomer (AI $\approx$ 1) ($F = 6.81$, df $= 5, 41$, $P < 0.001$; $F = 11.33$, df $= 5, 42$, $P < 0.001$; and $F = 4.93$, df $= 5, 36$, $P = 0.001$, respectively).

Individual Compounds and Their Mixture. The *RR* stereoisomer generated significantly greater EAG deflections at 10 ng than at 5 ng, whereas *SR* produced deflections of similar amplitude at both concentrations (Figure 5; $F = 17.63$, df $= 4, 84$, $P < 0.001$). The EAG amplitude due to 5 ng *RR* was higher than with 5 ng *SR*. A blend of 5 ng of each of these two compounds produced an intermediate EAG amplitude between 5 and 10 ng *RR*, but not significantly different from either one. The EAGs with *SR* were significantly wider than with *RR* or the blend ($F = 19.57$, df $= 4, 84$, $P < 0.001$). The *RR*–*SR* blend produced EAGs of intermediate width between *RR* and *SR*, but not significantly different from *RR*.

DISCUSSION

This study supports our previous findings that although the *RR* stereoisomer of supellapyrone is the natural pheromone of *S. longipalpa*, male antennae respond to both the *RR* and *SR* isomers (Leal et al., 1995). Under field conditions, males are attracted to low doses of *RR*, and to higher doses of *SR*. Olfactometer tests

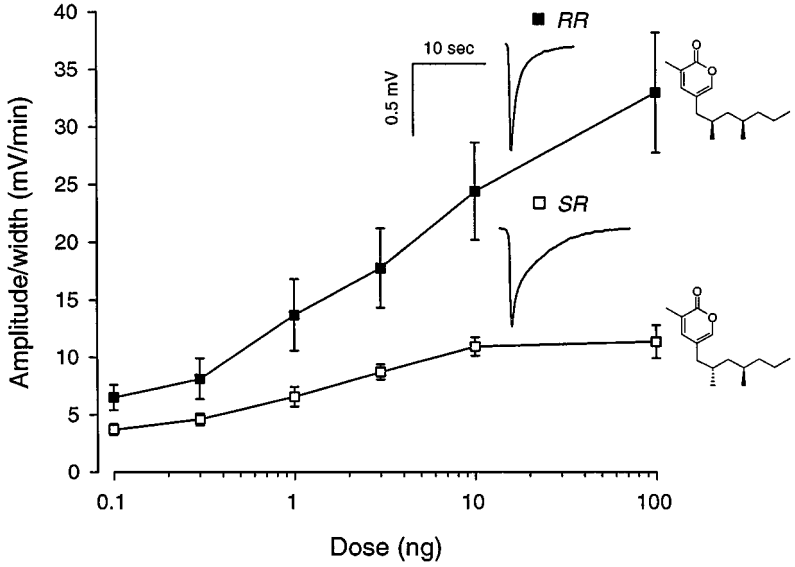

FIG. 3. Analysis of the shape of the EAG responses of male *S. longipalpa* antennae to *RR*- and *SR*-supellapyrone. EAG amplitude increases more than EAG recovery (width) with dose for both compounds, but this trend is steeper with *RR*. Also shown are two EAG responses to 10 ng of *SR* and *RR* delivered to the same antenna a few minutes apart.

revealed that males respond behaviorally to *SR* and *SS* also but at much higher doses than with the natural isomer. No response (behavioral or EAG) was elicited by *RS*. *RR* also induced a behavioral response in the highest percentage of males and the largest EAG amplitudes. Changing the methyl group farthest from the ring (position 4) from the (4′R)- to the (4′S)-configuration (i.e., *RR* to *RS*) resulted in complete suppression of the behavioral response. The antennae of males were, in fact, anosmic to *RS*, as determined by the lack of EAG responses to this isomer, except at exceptionally high concentrations. In contrast, changing the methyl group in position 2 from the (2′R)- to the (2′S)-configuration (i.e., *RR* to *SR*) reduced but did not eliminate the behavioral response. Interestingly, maintaining the syn-configuration by changing both methyl branches from *R* to *S* (i.e., *RR* to *SS*) did not result in the same dramatic reduction in behavior as changing from *RR* to *RS*. In fact, *SS* and *SR* induced behavioral responses in a similar percentage of the males, although *SS* produced significantly smaller EAGs. A 100-fold higher dose of *SR* was needed to stimulate behavioral responses of the same magnitude as *RR*. However, *RR* and *SR* produced similar EAG amplitudes. This last result showed a clear disparity between the antennal and the behavioral responses to the *SR*-isomer.

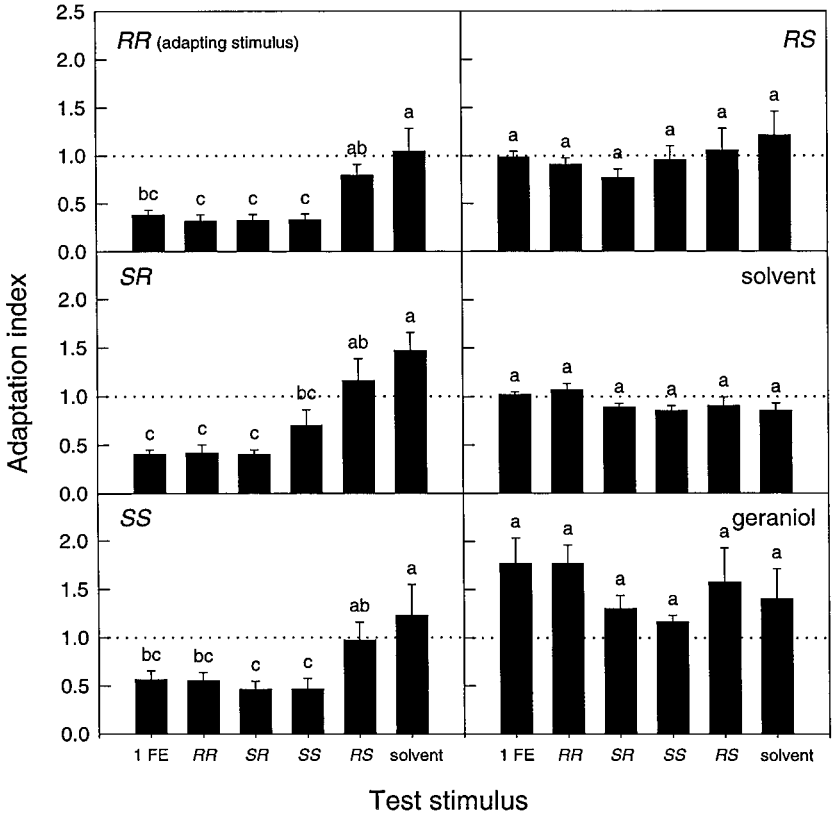

FIG. 4. Effect of adaptation of *S. longipalpa* male antennae on the responses to the stereoisomers of supellapyrone and to geraniol. Adaptation with *RR*, *SR*, and *SS* (left) caused a reduced EAG response (AI < 1) to 1 FE, *RR*, *SR*, and *SS*, but not to solvent or to *RS*. *RS*, solvent, and geraniol (right) failed to adapt the antenna to any of the test stimuli (AI ≥ 1).

Such a disparity between behavioral and EAG responses can be explained by an isomer acting as an antagonist of the natural pheromone. In the sympatric scarab beetles *Anomala osakana* Sawada (Osaka beetle) and *Popilia japonica* Newman (Japanese beetle), for instance, (*S*)-japonilure and (*R*)-japonilure, respectively, serve as the natural pheromones that release sexual responses in males (Tumlinson et al., 1977; Leal, 1996). However, because the (*R*)-enantiomer inhibits male response in the Osaka beetle, its antennae must recognize both isomers to accommodate such agonist–antagonist enantiomeric discrimination (Leal, 1998). In *S. longipalpa*, on the other hand, the nonnatural isomers do not appear to either suppress or promote male sexual responses. This is readily apparent in behavioral

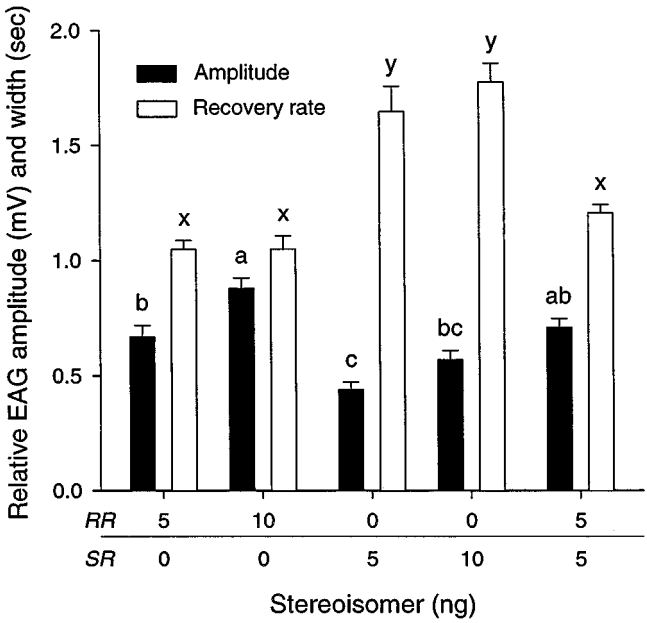

FIG. 5. EAG peak amplitude and width (mean + SE) in response to *RR*- and *SR*-stereoisomers and their blend. *SR*-supellapyrone produced the wider peaks and *RR* the highest amplitudes. The EAG amplitude of the blend was similar to that of *RR*. However, its width was reduced compared with the width of *SR* alone. Peak amplitude and width are standardized relative to the extract of a virgin female (1 FE). Different letters indicate significant differences ($P < 0.05$, Tukey's) for separate tests for amplitude and recovery time.

assays with mixtures of stereoisomers, which do not inhibit the response to *RR*-supellapyrone (Charlton et al., 1993) and in our preliminary experiments showing that the *SR*-isomer, the least active of the four stereoisomers, also does not diminish behavioral responses to *RR*-supellapyrone (personal observation). Further behavioral and trapping assays, especially with mixtures of the *RR*- and *SR*-isomers, are warranted before we may conclude unequivocally that attraction of males to the natural *RR*-supellapyrone is not modulated by its stereoisomers.

The *SR*- and *RR*-isomers give almost equivalent EAG responses, but 100-fold more *SR* is required for similar behavioral responses, so these two isomers could stimulate different olfactory receptor neurons, and this, in turn, could explain the disparity between EAG and behavior. EAG cross-adaptation has been used to test a similar hypothesis in other insect species (Dickens and Payne, 1977; Nagai et al., 1977; Lucas et al., 1994). In the American cockroach, *Periplaneta americana* (L.), cross-adaptation EAG results demonstrated that periplanone-A and periplanone-B

stimulate different receptor cell types (Nishino and Manabe, 1983; Tsuchiya and Takahashi, 1991), a finding corroborated by single cell recordings (Sass, 1983). Our data showed cross-adaptation between *RR* and *SR*, strongly suggesting that these two compounds stimulate the same odorant receptors. Hence, the hypothesis that *RR* and *SR* stimulate different receptor neuron types and that this results in different behavioral responses but similar EAGs, was not supported.

However, EAG cross-adaptation data must be interpreted with caution. Lack of cross-adaptation between two compounds is a clear indication that each interacts with different sensory neuron types tuned to specific compounds, but cross-adaptation may occur even if there are receptor neurons tuned to each of the compounds. For example, each receptor neuron type may respond predominantly to one of the odorants, but may exhibit some response to the other, sufficient to result in EAG cross-adaptation at relatively high doses. In the *S. longipalpa* system, however, the most likely explanation of cross-adaptation is that males possess only one type of pheromone receptor neuron because females produce only the *RR* isomer. Possibly, as discussed earlier, closely related sympatric species use one of the other isomers as a sex pheromone, and *S. longipalpa* males have receptors to detect it, and thus avoid mating with heterospecific females. This seems to be the case in *Periplaneta* cockroaches (Gemeno and Schal, 2003), but not in *S. longipalpa* because the behavioral tests do not indicate antagonism with the nonnatural isomers, but rather differential sensitivity to the four isomers of supellapyrone. Also, the addition of *SR* to *RR* did not attenuate the response to *RR*. However, the only definitive way to determine if each isomer stimulates the same or different receptor cells is to record from individual antennal receptor neurons.

If, as assumed, *SR*- and *RR*-supellapyrone do interact with the same receptor neurons, how do they produce similar EAGs, yet differ so dramatically in the behaviors elicited? The insect brain cannot discriminate between identical sensory inputs projecting from the same sensory neurons. Therefore, in order to elicit different behavioral responses, the two isomers must be discriminated within the sensillum. There are several ways in which this could be achieved (Kaissling, 2001). Once the hydrophobic pheromone molecule enters the sensillum, it must be transported to the neuronal receptors through the aqueous sensillum lymph. This transport is mediated by pheromone binding proteins (PBPs), which may exhibit high specificity for certain ligands. For example, in the gypsy moth, *Lymantria dispar* (L.), two PBPs differ in their enantiomeric binding preference: PBP1 has a higher affinity for (−)-disparlure while PBP2 has a higher affinity for the (+)-enantiomer (Plettner et al., 2000). The odor molecules must then interact with olfactory receptors at the neuronal plasma membrane, and also with pheromone-degrading enzymes, which clear the pheromone to favor further neuronal stimulation by new pheromone molecules. Any of these protein–ligand interactions can contribute to or diminish the specificity of response.

For example, could the differences in recovery rates (Figure 3) be related to different biochemical interactions of the *RR*- and *SR*-isomers with the sensory neurons? If so, could this explain the lack of correspondence between behavioral and EAG responses of the nonnatural *SR*-isomer? Differences in EAG recovery times have been reported in other insects (Guerin and Visser, 1980; Averill et al., 1988; Fescemeyer and Hanson, 1990; Light et al., 1992) and several hypotheses have emerged from early attempts to explain these differences. Recovery rates may reflect the amount of time required for the impulse frequency of individual neurons to return to the nonstimulated state (Whitehead, 1986), the amount of time that a stimulant molecule spends bound to receptors (Roelofs and Comeau, 1971), the response latency and response onset and offset of the neuron (Baker and Roelofs, 1976), the time required for deactivation of the molecules (Kaissling, 1974), the specificity of PBPs, receptor proteins and enzymes (Dickens et al., 1993), and compartmentalization of odorant molecules in the aqueous sensillum lymph (Dickens et al., 1993). To our knowledge, none of these hypotheses have been formally tested. We speculate, like previous authors, that differences in EAG recovery rates reflect differences in the intrasensillar processing of odorants. We further suggest that for similar compounds that activate receptor neurons of the same type, disparate EAG recovery rates could explain the lack of correspondence between EAG and behavior. Others have also noted that EAG recovery rates may correlate better with behavior than with EAG amplitude (Averill et al., 1988; Fescemeyer and Hanson, 1990). Whereas the EAG is rarely used to study intrasensillar events, its ability to discriminate, as we speculate, between isomers that may interact with the same receptors but elicit different behavioral responses adds substantial power to this time-tested procedure. It highlights the importance of quantifying more than one EAG parameter, especially in studies of closely related odorants.

One factor that should not be overlooked in dose–response studies is the purity of the synthetic compounds, especially when used at relatively high doses with highly sensitive antennae, as in the present study. The high purity (i.e., high enantiomeric excess) of our samples ensured that artifacts were kept to a minimum. Nevertheless, the nonnatural *SS*-isomer has 4.4% *RR* so that the behavioral response observed at high doses of *SS* may be elicited by the *RR* impurity, provided that there is no antagonism between them. Impurities, however, do not explain the EAG and behavioral response elicited by the *SR*-stereoisomer, which is completely devoid of any diasteromers, i.e, it does not contain any detectable amounts of *RR* or *SS*. Because males do not respond to *RS* at any dose, *RS* impurity should have no effect on response to *SR*. Also, lack of any response to *RS*, even at high doses, indicates that its minor contamination with *SR* did not contribute to either behavioral or EAG responses. Regardless, these considerations do not explain the disparity between EAG and behavior observed with *SR*.

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